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A LOW MOLECULAR WEIGHT ALKALINE PROTEINASE FROM *CONIDIOBOLUS CORONATUS*

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SUMMARY

An extracellular, low molecular weight alkaline proteinase (alkaline proteinase "B") has been purified to homogeneity from the culture filtrate of *Conidiobolus coronatus* (NCIM 1238). A 12-fold purification was achieved with a specific activity of 29,760 u/mg. The enzyme had an optimum pH and temperature of 9.7 and 45°C respectively. It was most active towards casein and had a molecular weight of 6,800, the lowest reported so far. It was stable between pH 6.5 - 7.5. Alkaline proteinase "B" is a serine proteinase. It showed an esterolytic activity on N-benzoyl-L-tyrosine ethyl ester (BTEE) and was successfully used to resolve the racemic mixture of D, L - phenylalanine and D,L - phenylglycine and can thus potentially replace subtilisin Carlsberg in resolving the racemic mixture of amino acids.

INTRODUCTION

Alkaline proteinases from several microbial sources have been purified and characterized. However, few reports exist on proteolytic enzymes from Entamophthorales and in particular, from *Conidiobolus.* Tokuyama and Asano (1978) reported alkaline proteinase from *Conidiobolus adiaereticus,* which has a molecular weight of 25,000. Partial purification of a proteinase from *Conidiobolus lamprauges* was reported by Ishikawa *et. al.* (1981). An alkaline proteinase from a *Conidiobolus* has been reported by Whitehill *et.al.* (1960). Recently we have reported that an isolate of *Conidiobolus* produces appreciable amounts of alkaline proteinase (Srinivasan *et. al.*, 1983). Subsequent studies showed that the culture broth

contains a high molecular weight alkaline proteinase (designated as proteinase 'A') and a low molecular weight alkaline proteinase (proteinase 'B').

In this paper, purification and characterization of a low molecular weight alkaline proteinase 'B' from a *C.coronatus* and its significance in resolving racemic mixtures of D,L - phenylalanine and D,L - phenylglycine is reported.

MATERIALS AND METHODS

Organism : *Conidiobolus coronatus* NCIM 1238, earlier designated as *Conidiobolus* sp. (NCL 82-1-1)(Srinivasan *et. al.*, 1983), was maintained on MGYP agar slopes (malt extract 3.0g, glucose 10.0g, yeast extract 3.0g, peptone 5.0g and agar 20.0g per litre) at 30°C.
Cultivation : For the production of enzyme, *C.coronatus* was grown in a medium containing 2% casein, as described earlier (Srinivasan *et. al.*, 1983) at 30°C on a rotary shaker at 200 rpm for 96h. Culture filtrate was obtained by filtering the broth through Whatman No. 1 filter paper.
Purification : Alkaline proteinase 'B' was purified from the culture filtrate using conventional methods like alcohol precipitation, DEAE-cellulose (OH^- form) treatment, ammonium sulphate precipitation and preparative PAGE at pH 7.6 (Zuidweg *et. al.* , 1972). The eluted enzyme was further purified on CM-cellulose column (1.2 x 15cm) equilibrated with 0.02 M potassium phosphate buffer, pH 7.0, and eluted with 0.06 M phosphate buffer, followed by gel filtration on Sephadex G-50 (Column 1.6 x 100cm). The active fractions were pooled and concentrated. The purity of the enzyme was tested on SDS-PAGE and analytical gel electrophoresis (pH 4.3 and pH 7.6).
Enzyme assay : The reaction mixture (2 ml) contained 10 mg of Hammarsten casein (Merck, FRG) in 0.1M sodium carbonate buffer, pH 9.7, and suitably diluted enzyme. The reaction was terminated after incubation for 20 min at 35°C by adding 3ml of 5% trichloroacetic acid and the acid soluble material was estimated at 280 nm after removing the precipitate by filtration. One proteinase unit is defined as the amount of enzyme which catalyzed release of 1μmole tyrosine per min.
The esterase activity of the enzyme was determined according to Walsh and Wilcox (1970). The activity of proteinase 'B' on azocoll, azocasein and ovalbumin was determined using the method of Ansari and Stevens (1983).
Protein determination : The protein was estimated by the dye-binding method (Bradford, 1976).
Resolution of racemic mixture of D,L- phenylalanine and D,L-phenylglycine : Resolution of D,L-phenylalanine and D,L-phenylglycine was carried out according to the procedure of Roper and Bauer (1983). Enzyme concentration employed in the reaction mixture was 200 μg for 221mg of the respective racemic mixtures. The pH was controlled using an automatic pH Stat (Radiometer, Copenhagen, Denmark) at pH 7.5.

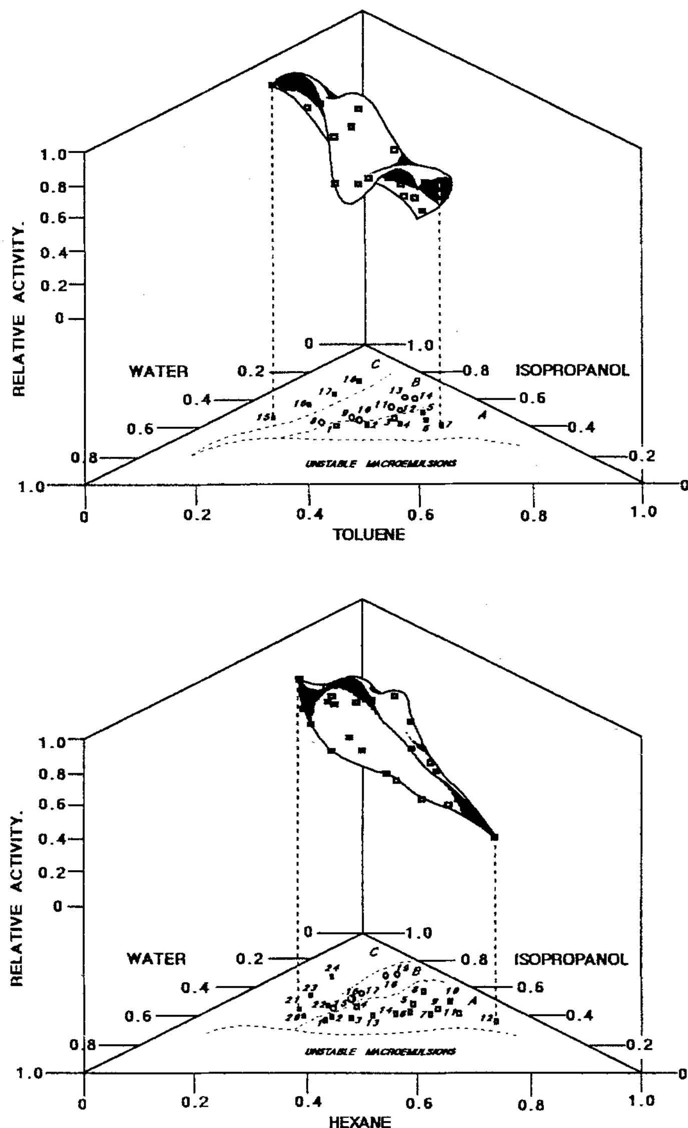

Fig.3. Dependence of PPO activity on the composition of toluene- (upper graph) and hexane-based (lower graph) systems expressed in molar fractions. The data and abbreviations used are given in Table 2.

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it was completely inhibited by phenyl methyl sulfonyl fluoride (PMSF) (0.1mM) and diisopropyl phosphofluoride (DFP) (0.3mM), whereas *p*-chloromercury benzoate (PCMB) was not inhibitory. Ethylene diamine tertraacetic acid (EDTA) (20mM) had no effect on the activity indicating non-involvement of any metal ions, whereas, the enzyme from *C.adiaereticus* lost 23% activity by 1mM EDTA (Tokuyama and Asano, 1978).

Resolution of racemic mixtures : The enzyme showed esterolytic activity on BTEE. The Km and Vmax of the enzyme with BTEE were 0.25mM and 1,425 s^{-1} $mole^{-1}$, respectively. Proteinase 'B' was successfully exploited for the resolution of racemic mixtures of D,L-phenylalanine and D,L-phenylglycine. The enzyme specifically hydrolysed the ester bond at the carboxyl group of the (L) isomers of derivatised (N-acetylated and C-esterified) phenylalanine and phenylglycine with yields of 82% and 78% respectively. The esters of (D) isomers remained unaffected by the enzymatic treatment. Therefore, the alkaline proteinase 'B' from *Conidiobolus* sp. can replace subtilisin Carlsberg (Roper and Bauer, 1983), which is presently being used in the industry for resolution of D,L-isomers.

TABLE 1 : Purification of alkaline proteinase 'B' from *C.coronatus*

Step	Total Protein (mg)	Total Units Ux10³	Specific activity	Purification fold
Culture broth	8,100	20,100	2,480	1.0
Alcohol precipitation	1,850	14,900	8,054	3.3
DEAE-cellulose (negative adsorption)	574	12,100	21,080	8.5
Ammonium sulphate precipitation (90% saturation)	450	10,050	22,320	9.0
Preparative PAGE Proteinase 'B'	55	1,370	24,900	10.0
CM-cellulose chromatography	33	902	27,330	11.2
Sephadex G-50 gel filtration	25	744	29,760	12.0

All operations were carried out at 4°C.

TABLE 2 : Physico-chemical properties of alkaline proteinase from various *Conidiobolus* sp.

Organism	Molecular weight	pI	Optimum pH	Optimum temp (°C)
C.coronatus				
Proteinase 'B'[1]	6,800	8.5	9.7	45
Proteinase 'A'[2]	22,000	9.2	10.0	45
Conidiobolus sp.[3]	30,000	10.2	9.0	40
C.adiaereticus[4]	25,000	8.1	9.0	40

1. Present work
2. Ghadge G D (1986)
3. Whitehill *et. al.* (1960)
4. Tokuyama and Asano (1978)

TABLE 3 : Specific activity of alkaline proteinase 'B' on various substrates

Substrate	Specific activity U/mg
Casein	29,750
Haemoglobin	17,500
Azoalbumin	18,900
Azocasein	19,750
Azocoll	25,250
BSA	2,100
Ovalbumin	500

Under identical assay conditions

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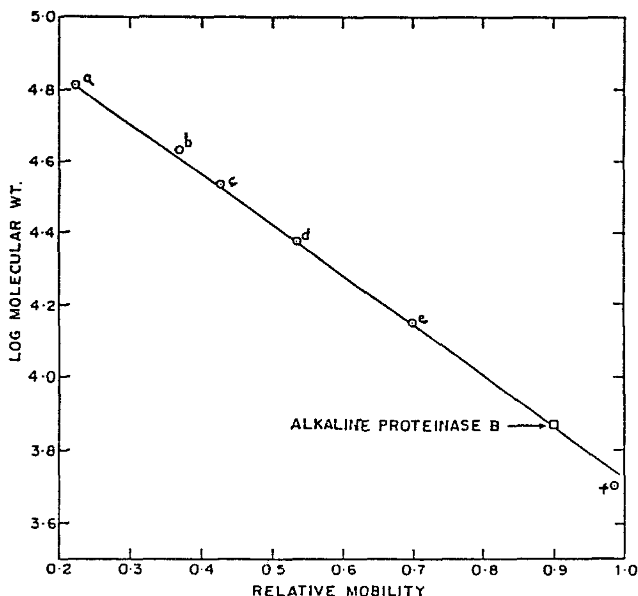

Fig. 1 : Molecular weight determination of alkaline proteinase "B" by SDS-PAGE. a. BSA (65 kd), b. Ovalbumin (43 kd), c. Pepsin (34 kd), d. Trypsinogen (24 kd), e. Lysozyme (14.3 kd), f. Trypsin inhibitor (5 kd).